BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
14 (2018) 1823–1832

Research Article

nanomedjournal.com

Early detection of pancreatic cancers in liquid biopsies by ultrasensitive fluorescence nanobiosensors

Madumali Kalubowilage, PhD[a,1], Obdulia Covarrubias-Zambrano, BSc[a,1], Aruni P. Malalasekera, PhD[a,1], Sebastian O. Wendel, PhD[a,b,1], Hongwang Wang, PhD[a], Asanka S. Yapa, PhD[a], Lauren Chlebanowski, BSc[a], Yubisela Toledo, BSc[a], Raquel Ortega, BSc[a], Katharine E. Janik, BFA[a], Tej B. Shrestha, PhD[b], Christopher T. Culbertson, PhD[a], Anup Kasi, MD[c], Stephen Williamson, MD[c], Deryl L. Troyer, PhD, DVM[b], Stefan H. Bossmann, PhD[a,*]

[a]*Department of Chemistry, Kansas State University, Manhattan, KS, USA*
[b]*Department of Anatomy & Physiology, Kansas State University, Manhattan, KS, USA*
[c]*University of Kansas Medical School, Kansas City, KS, USA*

Received 7 March 2018; accepted 24 April 2018

Abstract

Numerous proteases, such as matrix metalloproteinases (MMPs), cathepsins (CTS), and urokinase plasminogen activator (UpA), are dysfunctional (that is, over- or under-expressed) in solid tumors, when compared to healthy human subjects. This offers the opportunity to detect early tumors by liquid biopsies. This approach is of particular advantage for the early detection of pancreatic cancer, which is a "silent killer". We have developed fluorescence nanobiosensors for ultrasensitive (sub-femtomolar) arginase and protease detection, consisting of water-dispersible Fe/Fe_3O_4 core/shell nanoparticles and two tethered fluorescent dyes: TCPP (Tetrakis(4-carboxyphenyl)porphyrin) and cyanine 5.5. Upon posttranslational modification or enzymatic cleavage, the fluorescence of TCPP increases, which enables the detection of proteases at sub-femtomolar activities utilizing conventional plate readers. We have identified an enzymatic signature for the detection of pancreatic adenocarcinomas in serum, consisting of arginase, matrix metalloproteinase-1, -3, and - 9, cathepsin-B and -E, urokinase plasminogen activator, and neutrophil elastase, which is a potential game-changer.

Key words: Nanomedicine; Nanodiagnostics; Liquid biopsy; Biophotonics; Pancreatic cancer detection; Pancreatic ductal adenocarcinoma; Sub-femtomolar limit of detection

Pancreatic cancer is the third leading cause of cancer-related deaths in the United States, with a 5-year survival rate of less than 6%. In 2017, the U.S. alone there were an estimated 55,440 new cases of pancreatic cancer and 44,330 deaths.[1] Detection is usually accomplished in an advanced-stage, leading to a very poor prognosis. It is well established that surgical treatment for liver metastases from pancreatic cancer cannot offer long-term survival benefit for the vast majority of patients.[2] Additionally, the outcomes of radiotherapy and chemotherapy are equally unfavorable.[3] Pancreatic ductal adenocarcinoma (DAC, 85%-

Abbreviations: AJCC, American Joint Commission on Cancer; MMP, matrix metalloproteinase; CTS, cathepsin; UpA, urokinase-type plasminogen activator; PBS, phosphate-buffered saline; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); FRET, Förster Resonance Energy Transfer; SET, dipole-surface energy transfer; DAC, pancreatic ductal adenocarcinoma; MAC, metastatic adenocarcinoma; NET, pancreatic neuroendocrine tumors; MNET, metastatic neuroendocrine tumors.

This work was funded by NSF (CBET 1159966 and 1337438) and the Johnson Cancer Center at Kansas State University. This research is supported in part by the Vendig Cancer Research Fund, established in 2016 by 1952 K-State alumnus Lee Vendig and his son, Lee Vendig II.

The authors declare no competing interest.

*Corresponding author at: Kansas State University, Department of Chemistry, Manhattan, KS 66506-0401m.
E-mail address: sbossman@ksu.edu. (S.H. Bossmann).

[1] These authors have contributed equally.

https://doi.org/10.1016/j.nano.2018.04.020
1549-9634/

90% of all cases) is characterized by desmoplasia.[4] The accumulation of extracellular matrix (ECM) components alters the architecture of pancreatic tissue causing abnormal configurations of blood and lymphatic vessels leading to poor perfusion.[4] This effect is ultimately responsible for the inefficacy of classic chemotherapy against DAC.[4] Based on data from the National Cancer Data Base (1992–2004, statistics last revised on 09/15/2016), the 5-year observed survival for exocrine pancreatic cancer (predominantly DAC) is 14% when the cancer was discovered at stage IA, 12% at IB, 7% at IIA, 5% at IIB, 3% at III, and 1% at stage IV.[5] All stages used here were defined in accordance with the American Joint Committee on Cancer (AJCC) TNM staging system.[6] Based on these facts, the development of a feasible early detection tool for pancreatic cancer would have a significant impact on saving lives.[2]

Liquid biopsies

Detecting cancer and other diseases by means of a simple blood test has become a realistic possibility. Virtually all competing companies, among them Personal Genome Diagnostics,[7] Genomic Health,[8] Myriad Genetics,[9] Guardant Health[9] and Pathway Genomics[9] rely on PCR to detect genetic mutations, and various RNAs that are overexpressed in tumors. Earlier clinical blood tests for pancreatic cancer have focused on CA19-9 (carbohydrate antigen 19-9),[10] CEA (carcinoembryonic antigen)[11] and recently K-ras gene mutations[12] to date. CancerSEEK,[13] the most developed pancreatic cancer test to date, evaluates plasma-levels of 8 cancer indicating proteins and the presence of mutations in 2001 genomic positions for detecting 8 solid tumor types, among them pancreatic cancer with an overall percentage of cancer detection of 75 percent. The majority of pancreatic cancer patients were diagnosed at AJCC stage II. Due to the unfavorable survival statistics, pancreatic cancer has to be diagnosed as early as possible.

The approach discussed here focuses on detecting the protease/arginase[14–16] signature of solid tumors. Including blood tests for the estimated 14.5 million cancer survivors in the United States, the market potential for liquid biopsies is currently estimated to more than $20 billion a year. The average genomic test is currently > $5000, which will prove prohibitively expensive for many patients. Compared to the state-of-the-art in liquid biopsies, protease profiling using the proposed approach will result in significantly reduced costs: $100 to $200 per protease/arginase profile for the end-user appears to be realistic. It should also be noted that there is a high potential for synergy between genomic and proteomic tests: genetic tests often show the potential for disease development, but not exactly when the transition to a tumor actually occurs. Protease/arginase assays can accurately detect that transition.

Proteases and arginase as cancer biomarkers

Matrix metalloproteinases (MMPs), serine proteases and cysteine proteases, as well as arginase, have well-documented roles in malignant progression and immune (dis)regulation in cancer.[17–19] It is of importance that tumor-promoting proteases act as a part of an extensive multidirectional network of proteolytic interactions. There are 570 known human proteases, coupled with a smaller group of endogenous protease inhibitors that tightly regulate their activity.[20] In general, cathepsin B, urokinase plasminogen activator (UpA), metalloproteinases (MMP) occupy central nodes for amplifying proteolytic signals passing through the network.[17] Recent research has shown that this proteolytic signaling network interacts with other important signaling networks, such as chemokines, cytokines, and kinases.[17] Understanding this extensive network of proteolytic interactions as a system of activating and inhibiting reactions may prove to be an important key to unlock tumor biology.

Gene expression analysis

Gene expression analysis[21] is a straightforward approach to determine the proteases that are overexpressed in solid tumors, such as pancreatic cancer. A wealth of data is available from databases, such as NCBI GEO, Entrez Gene ID, Unigene ID and Gene Symbol.[21] This strategy is able to select enzyme candidates that have a high probability of being proximal biomarkers for pancreatic cancer from the human genome.[20] This makes the selection process far less arbitrary than it would have to be, based on cancer literature alone.

The relevant datasets for this study were obtained from the publicly accessible NCBI GEO database.[22] Criteria for datasets included in the analysis were that the investigated species is *Homo sapiens* and that the dataset contains samples from both primary tumor samples and healthy human tissue.

Methods

Statistical analysis

The fold change of gene expression is taken as an indicator of the up- or downregulation of the genes of interest. The R software environment for statistical computing was used to extract the relevant raw data, calculate *P* values[23] and generate boxplots to illustrate data-ranges.[24,25] A *P* value (calculated probability) is defined as the probability of finding the observed results if the null hypothesis (H_0) of a study question is true.[26] In common terms, the null hypothesis assumes no difference between a property in the group of study subjects vs. a control group.[26] The term "Fold Change" (FC) is defined as the "ratio of the difference between final value B and the initial value A over the initial value. FC = (B − A)/A. logFC is the decadic logarithm of FC.[27]

Nanobiosensor synthesis

The synthesis of the nanobiosensors has been performed according to established and published procedures.[14–16] In short, water-dispersible Fe/Fe_3O_4 nanoparticles featuring dopamine ligands,[28] TCPP,[14] and cyanine 5.5[14] were synthesized according to established procedures. Details are provided in the SI section.

For all MMPs and cathepsins employed here, sub-femtomolar limits of detection (LOD) have been realized.[14,15] After optimization, ten repetitions of the calibration procedure

established a relative error under 2%, which is sufficient for clinical applications.[14,15]

Serum samples for liquid biopsy

All biospecimens were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30] Group sizes: apparently healthy volunteers: n = 48, patients with pancreatitis: n = 4, pancreatic ductal adenocarcinoma (DAC): n = 9, metastatic adenocarcinoma (MAC): n = 9, pancreatic neuroendocrine tumors (NET): n = 5, metastatic NET (MNET): n = 2, all pancreatic cancers (ALL): n = 35. The groups of cancer patients and healthy volunteers were age- and gender-matched.

Fluorescent plate reader measurements: calibration and validation

A BioTek Synergy 2 plate reader (tungsten halogen lamp, excitation bandpass filter: 421 ± 10 nm, analysis bandpass filter: 650 ± 25 nm) with 96-well plates was used. Solution (1) consisted of HEPES buffer (25 μmol) (2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid) and was prepared enriched with Ca(II), Mg(II), and Zn(II) (10 μmol each) at 298 K (pH = 7.2) to ensure full enzymatic activities. Solution (2) containing the Fe/Fe_3O_4 based nanobiosensor was prepared by dissolving 0.30 mg of the selected nanobiosensor in 1.0 mL of HEPES buffer by sonication for 10 min at 298 K. The following samples were prepared and plated by adding solution (1) or solution (2) to 5 μL of serum sample; A: Sample Control (125 μL of solution (1) + 5 μL serum sample); B: Assay (125 μL of solution (2) + 5 μL of calibration solution containing known concentrations of purchased enzymes; C: Assay Control (125 μL of solution (2) + 5 μL of solution (1)); and D: Blank (130 μL of solution (1)). Each sample (total 130 μL) was loaded into one well a 96-well plate with at least three replicates of each assay per serum sample. Solutions were incubated at 310 K for 60 min, followed by detection of nanoplatform fluorescence at 298 K utilizing a 96-well fluorescence plate reader. Matrix effects have been previously evaluated by using heat-inactivated combined sera from the control group of healthy volunteers.[15] Heat inactivation of serum was performed according to established procedures.[31] The results of the calibration procedures are summarized in the SI section. However, the main focus of this study was on developing a quick fluorescence plate-reader method for pancreatic cancer detection, which will work reliably in a clinical setting. Therefore, the actual fluorescence signals measured by the plate reader were used to calculate the results discussed below.

Results

Diagnostic strategy in liquid biopsies

Our diagnostic strategy is based on the paradigms that a) protease networks in pancreatic cancer are dysfunctional, and b) unique protease/arginase signatures exist for many types of solid tumors. Therefore, Gene Expression Analysis was used as a selection tool to determine the most likely candidates. In Table 2, the IDs, *P* values[23] and logFC (down- or up-regulation of genes) for the group of target proteases in pancreatic cancer tissue samples are summarized.[24,25] It is of importance that these data were obtained by comparing the protease expression levels in primary pancreatic tumors using apparently healthy tissue samples from the same patients as controls. Since cancer is a systemic disease, however, it cannot be expected that all of these correlations can be verified by measuring the activity of proteases in a group of pancreatic cancer patients with a group of age- and gender-matched healthy volunteers. Much more likely, also noncancerous tissue will be affected by a tumor somewhere in the patient's body, because this tumor is connected to the blood supply starting from stage I.[17,32,33] (See Table 1.)

Table 1
Consensus sequences used in nanobiosensors employed.[29]

Nanobiosensor	Oligopeptide Tether
Arginase	GAG**RRRRRRR**AG
Cathepsin B (CTS B)	GAG**SLLKSR-MVPNFN**AG
Cathepsin E (CTS E)	GAG**EVAL-VALK**AG
MMP 1	GAG**VPMS-MRGG**AG
MMP 3	GAG**RPFS-MIMG**AG
MMP 9	GAG**VPLS-LYSG**AG
UpA	GAG**SGR-SA**G
Neutrophil Elastase	GAG**GEPV-SGLP**AG

Synthesis and validation of ultrasensitive nanobiosensors for protease and arginase

A detailed account of the development of Fe/Fe_3O_4-nanoparticle based diagnostic nanobiosensors is given in references.[14–16,34] Fe/Fe_3O_4 nanoparticles were synthesized by thermal decomposition of $Fe(CO)_5$.[14,35,36] The nanoparticles had a well-defined core/shell structure, with an average Fe(0) core diameter of 13 +/− 0.5 nm and Fe_3O_4 shell thickness of 2.0 ± 0.5 nm, respectively.[14] Dopamine formed a robust organic coating on the particle with a binding constant on the order of 10^{15} L mol^{-1}.[28] It also increased the water-solubility of the resulting nanoplatforms to >5 g L^{-1}.[28] Porphyrins were used as cleavable fluorescent dyes, because their photophysical properties are well characterized.[37] Cyanine 5.5 has been co-attached as a FRET quencher due to its large molar extinction coefficient.[38] Figure 1 shows the structure of the nanoplatform comprised of dopamine-coated Fe/Fe_3O_4, consensus sequence, TCPP, and Cy 5.5. In Table 2, the consensus sequences that were employed for detecting the selected proteases,[27] as well as the peptide tether for measuring arginase,[16] are summarized. Cyanine 5.5 is permanently linked to dopamine without using an enzyme-cleavable tether. The optimal average density of cyanine 5.5, which is directly bound to the dopamine units, and TCPP, which is tethered via oligopeptide, was determined to be 50+/−4 (Cy 5.5) and 35+/−3 (TCPP) per nanoparticle following a random-deposition based modeling approach[39] assuming a core/shell structure with an average Fe(0) core diameter of 13 +/− 0.5 nm and an Fe_3O_4 shell thickness of 2.0 ± 0.5 nm. The nanoplatforms were activated via enzymatic cleavage or

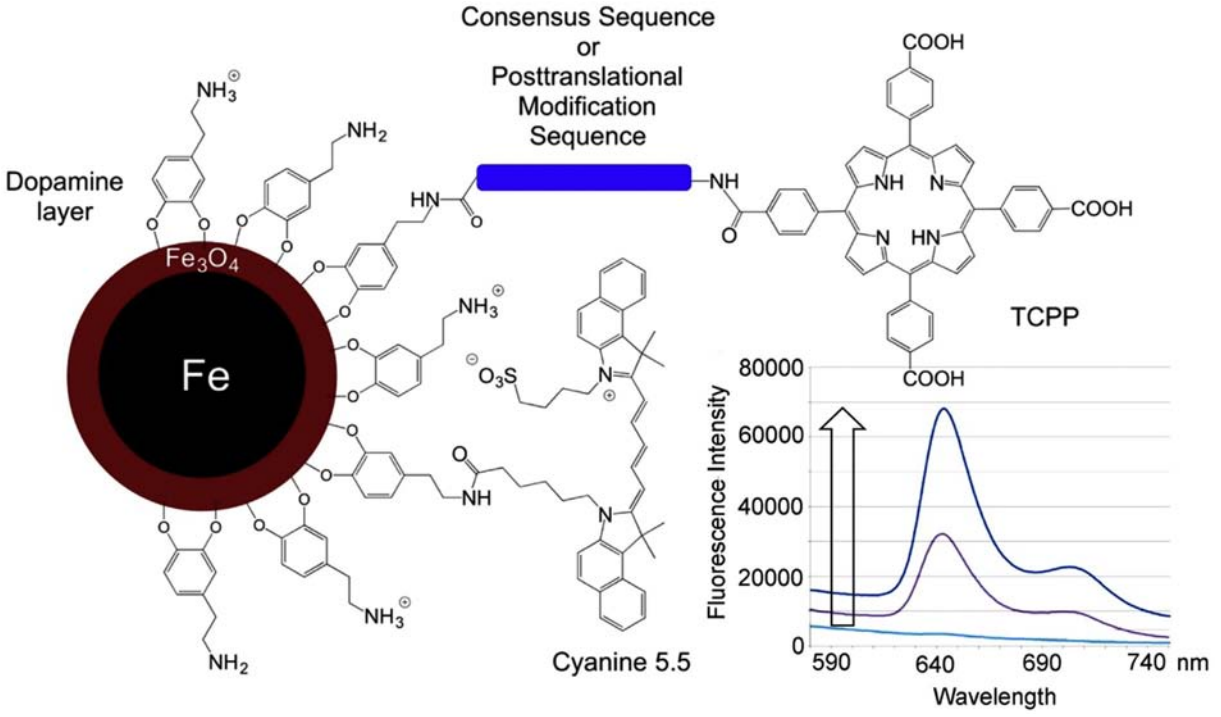

Figure 1. Chemical structure of the nanobiosensors for protease and arginase detection. The core of the nanobiosensor consists of dopamine-coated a Fe/Fe_3O_4 core to which 50+/−4 cyanine 5.5 and 35+/−3 TCPP molecules are bound, following a random-deposition based modeling approach.[39] The consensus sequences experience either proteolytic cleavage by their respective proteases, or the chemical constitution of the posttranslational modification sequence is changed. For instance, arginases I + II convert arginine to ornithine without proteolytic cleavage of the oligopeptide.[16] Inset: The fluorescence occurring from the nanobiosensors increases with incubation time.[14,15] This enables fluorometric detection of protease/arginase activities.

Table 2
NCBI GEO IDs, *P* values[23] and logFC (down- or up-regulation of genes) for the group of target proteases in pancreatic cancer tissue samples.[24,25]

ID	*P* value	logFC	Gene symbol	Gene title
Arginase 2				
7975268	4.20E-02	0.17303	ARG2	arginase 2
Cathepsin B				
8149330	3.18E-07	0.848623	CTSB	cathepsin B
Cathepsin D				
7945666	5.64E-07	0.534505	CTSD	cathepsin D
Cathepsin E				
7909164	6.00E-15	2.684101	CTSE	cathepsin E
UpA				
7928429	2.54E-10	1.374367	PLAU	plasminogen activator, urokinase
MMP1				
7951271	1.96E-04	1.225159	MMP1	matrix metallopeptidase 1
MMP3				
7951284	1.93E-05	0.66726	MMP3	matrix metallopeptidase 3
MMP9				
8063115	5.12E-09	1.116676	MMP9	matrix metallopeptidase 9
Neutrophil Elastase				
8024056	8.87E-02	−0.09965	ELANE	elastase, neutrophil expressed

posttranslational modification of the tether between central nanoparticle and dye, which led to increased TCPP-fluorescence (*light switch effect,* Figure 1).[14] Calibration and validation of the Fe/Fe_3O_4-based nanobiosensors were reported in reference.[15] (See Figs. 2–5.)

Protease signature of all pancreatic cancers

Gene expression analysis revealed upregulation of arginase, urokinase plasminogen activator (UpA), matrix metalloproteinase (MMP)-1, -3, and -9, cathepsin (CTS)-B, -D, and -E, as well

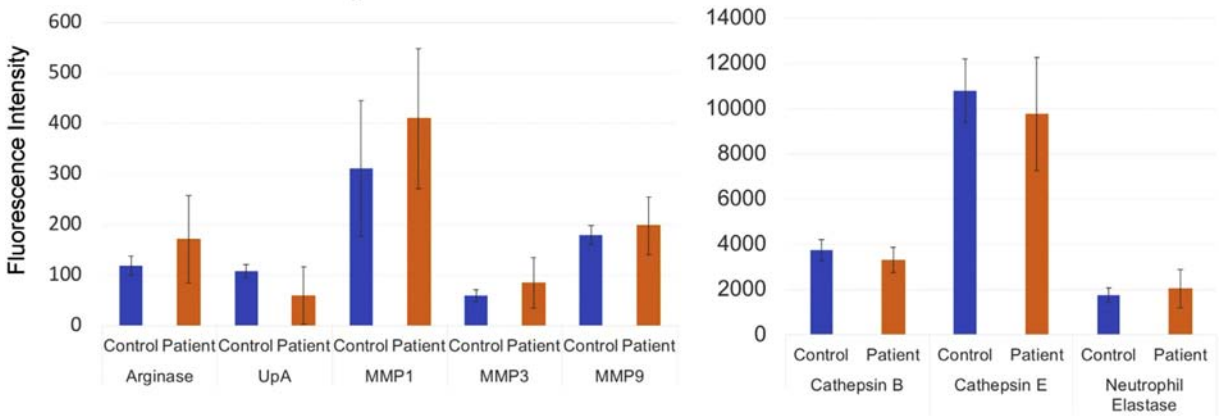

Figure 2. Bar graph (showing means and standard deviations for arginase and UpA, MMP-1, -3, and -9, CTS-B, -E, and NE); Group sizes: apparently healthy volunteers: n = 48, pancreatic cancers: n = 35; all samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30]

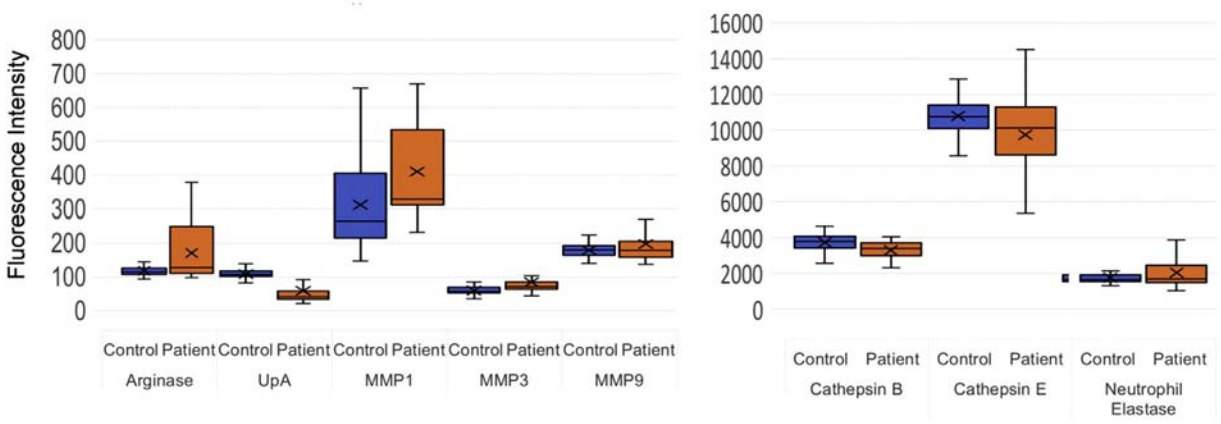

Figure 3. Box plots (indicating the observed data range) for arginase and UpA, MMP-1, −3, −9, CTS-B, −E, and NE; Group sizes: apparently healthy volunteers: n = 48, pancreatic cancers: n = 35; all samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30]

as the downregulation of neutrophil elastase (NE) expression in tumor tissue. The underlying paradigm of this study is that arginase and proteases that are overexpressed in tumor/stromal tissue can be detected in serum. For arginase, MMP-1, and MMP-3, significantly higher enzyme activities ($P < 0.05$[23]) were detected for the group of pancreatic cancer patients, compared to the age- and gender-matched control group. The enzymatic activity of MMP-9 was upregulated as well in the pancreatic cancer group, albeit not significantly ($P = 0.06696$). NE was also found to have higher activity ($P = 0.07838$), which was in disagreement with the predictions from gene expression analysis. For both, MMP-9 and NE, the calculation of the P values was affected by the relatively small numbers in pancreatic cancer and control groups.[23] The activity of cathepsin-D in serum in both groups was essentially the same ($P = 0.27854$). The activities of UpA, and CTS-B and CTS-E were significantly lower in the serum of cancer patients vs. the control group. A possible reason why the prediction of gene expression analysis and the measurements of protease activity in serum differ is that all proteases investigated here are biosynthesized as zymogens,[33] which then require proteolytic activation. This essential step may or may not be effective in tumor tissue (Figures 2 and 3).

Protease signature of pancreatic ductal adenocarcinomas (DACs)

Ductal adenocarcinomas have the highest incidence of all pancreatic cancers.[1] Considering the currently available options for pancreatic cancer treatment, detection of DAC has to be achieved at the earliest stage in order to be of significant help to the cancer patients. For this purpose, we have compared the enzymatic activities in the serum samples of the sub-group of 9 patients with DAC with a control group of 9 age- and gender-matched healthy volunteers. For DAC, the activities of arginase, UpA, MMP-1, -3, -9 and NE were upregulated, albeit only UpA and MMP-3 were statistically significant, owing to the small group sizes (n = 9).[23] Arginase was borderline significant ($P = 0.071938$). CTS-B and CTS-E showed downregulated activities for the DAC group, albeit not significant. The important finding from this study is that, even at small group sizes, UpA and MMP-3 were able to identify patients with ductal adenocarcinoma in a simple blood test (Figures 4 and 5)!

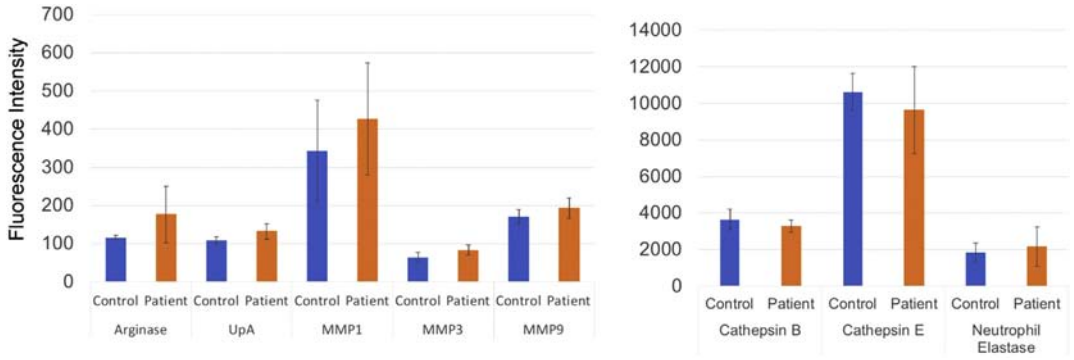

Figure 4. Bar graph (showing means and standard deviations for arginase and UPA, MMP-1, -3, -9, CTS-B, -E, and NE); Group sizes: apparently healthy volunteers: n = 9, pancreatic ductal adenocarcinoma (DAC): n = 9; all samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30]

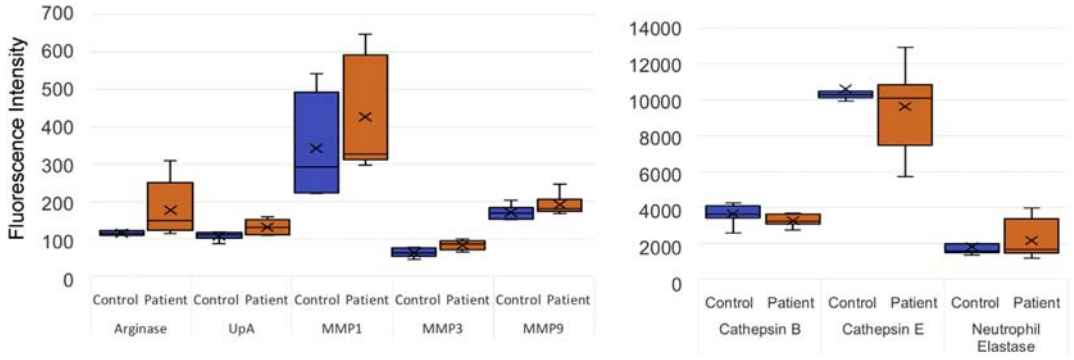

Figure 5. Bar graph (showing means and standard deviations) for arginase and UPA, MMP-1, -3, -9, CTS-B, -E, and NE; Group sizes: apparently healthy volunteers: n = 9, pancreatic ductal adenocarcinoma (DAC): n = 9; all samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30]

Do pancreatic cancer and pancreatitis have different protease signatures?

An important question with respect to establishing a routine test for (early) pancreatic cancer is, whether pancreatitis and pancreatic cancer can be distinguished by means of a simple blood test. The comparison of the protease signatures of pancreatitis (n = 4) and an age- and gender-matched group of pancreatic cancer patients (n = 9) is shown in Figure 6. According to our preliminary findings, the activities of CTS-B (down), MMP-9 (up), NE (down), and UpA (up) are significantly different for patients with pancreatitis, when compared to pancreatic cancer. Therefore, both conditions can be distinguished by means of a liquid biopsy. This finding shows that screening for pancreatic cancer in serum by means of measuring the activity of signature proteases is a feasible strategy.

Does pancreatitis interfere with pancreatic cancer detection?

With respect to the diagnosis of pancreatic cancer by means of a liquid biopsy, it is of importance that the protease expression patterns of patients with pancreatitis and apparently healthy patients are different, as indicated by our preliminary experiments that are summarized in Figure 7. The activity of arginase, MMP-1, MMP-3, and MMP-9 was significantly ($P < 0.05$)[23] higher in patients diagnosed with pancreatitis than in the age- and gender-matched control group. CTS-B and NE were significantly lower in activity.

Discussion

Although some patients in the sub-group of ductal adenocarcinoma (DAC) were characterized by high cathepsin D activities in serum, no statistically significant differences between both pancreatic cancer groups (all pancreatic cancers and DAC) and their respective control groups have been detected. This is surprising, because gene expression analysis predicted significant over-expression of cathepsin D in pancreatic tumor tissue. This may be an indication that for cathepsin D, there is no good correlation between activity in tumor tissue and in blood. Another reason for the observed discrepancy may be that all proteases are translated as zymogens (inactive enzymes). They require enzymatic activation, usually by another protease, thus forming a network.[17] Therefore, cathepsin D, as well as some of the other proteases, which do not fit the predicted pattern, may be synthesized in high concentration, but not activated.[17,40,41]

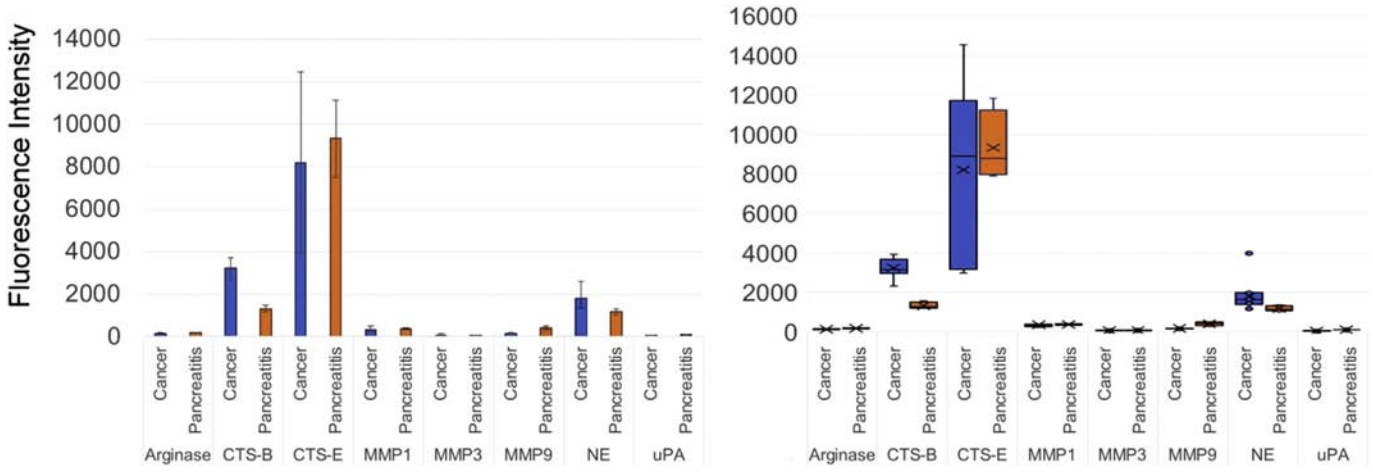

Figure 6. Bar graphs (showing means and standard deviations) and Box plots (indicating the observed data range) for arginase and UpA, MMP-1, -3, -9, CTS-B, -E, and NE; Group sizes: patients with pancreatitis: n = 4, pancreatic cancer patients: n = 10; all samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30]

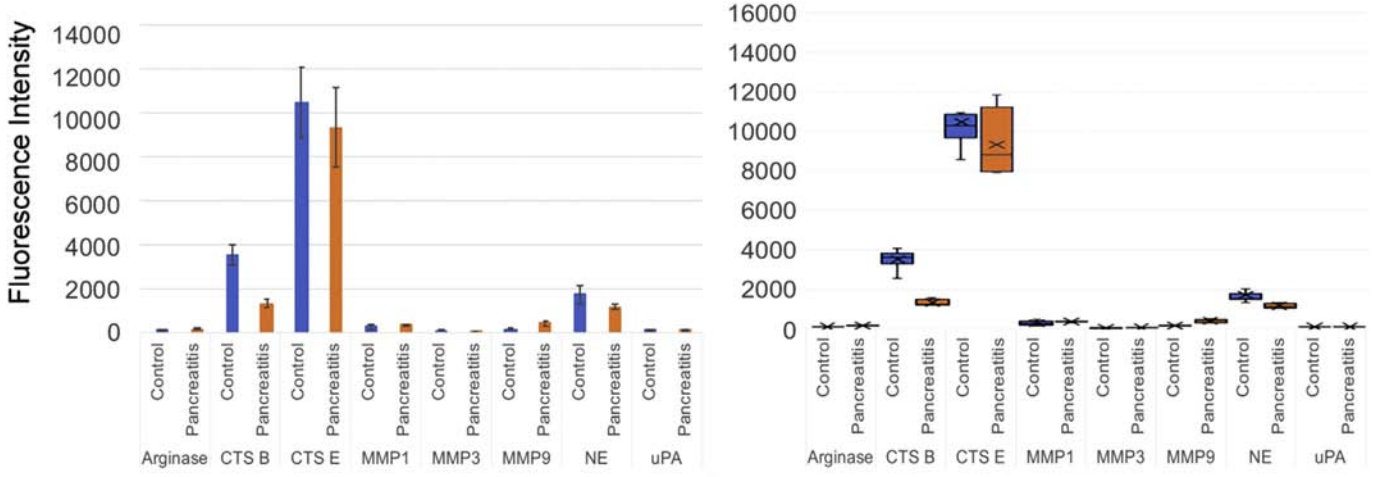

Figure 7. Bar graphs (showing means and standard deviations) and Box plots (indicating the observed data range) for arginase and UPA, MMP-1, -3, -9, CTS-B, -E, and NE; Group sizes: patients with pancreatitis: n = 4, healthy control group: n = 10; all samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30]

Arginase activity was higher in patients with pancreatic cancer and the sub-group of patients with ductal adenocarcinomas (DAC) when compared with the control group. However, the observed variations are much higher in both groups of pancreatic cancer patients than in the apparently healthy control groups. Cathepsin B expression was lower in both investigated pancreatic cancer groups (all pancreatic cancers and DAC). Gene expression analysis predicted upregulation of cathepsin B expression, which was not observed in this study. Genetic expression analysis also predicted that cathepsin E will be an excellent marker for pancreatic cancers and over-expressed in tumor tissue. However, in both pancreatic cancer groups (all pancreatic cancers and DAC) cathepsin E activity was significantly lower than in the respective control groups. Therefore, cathepsin E is a valuable member of the panel of proteases designed for early diagnosis of pancreatic cancer, but does not show the predicted upregulation. Gene expression analysis correctly predicted the upregulation of urokinase plasminogen activator (UpA). UpA's activity is enhanced in both pancreatic cancer groups (all pancreatic cancers and DAC), compared to their respective control groups. However, there is a considerable variability of UpA activity. Since UpA has numerous functions within the human body,[17] it is not surprising that its expression pattern varies, to a degree, between different human subjects. However, UpA may play a more important role with regard to early pancreatic cancer detection when data obtained from larger patient groups will become available.

Gene expression analysis also predicted overexpression of MMP-1 in pancreatic tissue. In this case, we were able to find enhanced MMP-1 activity in virtually all serum samples from pancreatic cancer patients. For the group of ductal adenocarcinomas MMP-1 is a proximal biomarker. In agreement with gene

Table 3
Significance table of pancreatic cancers and pancreatitis vs. control groups. **PAN:** all Pancreatic Tumors, **DAC:** Pancreatic Ductal Adenocarcinoma, **PCT:** Pancreatitis, **H:** Healthy Control Group.

	ARG	CTS B	CTS D	CTS E	MMP-1	MMP-3	MMP-9	uPA	NE
PAN	0.00112	0.00035	0.2785	0.0296	0.00179	0.00669	0.0670	1.1E-05	0.07084
DAC	0.07194	0.1624	0.3742	0.3408	0.28476	0.01381	0.1148	0.02463	0.47909
PCT vs. PAN	0.151349	1.4E-07		0.5019	0.73566	0.86827	0.01436	3.56E-05	0.03662
PCT vs. H	0.01847	1.2E-08		0.3216	0.06316	0.00776	0.01437	0.19038	0.00465

expression analysis, MMP-3 activities are enhanced in both groups of pancreatic cancer patients (all pancreatic cancers and DAC). MMP-9 activity was enhanced in both pancreatic cancer groups as well, which is in agreement with the prediction from gene expression analysis. However, MMP-9 activities detected in the serum of cancer patients and healthy volunteers were not statistically significant. They were found to be significantly higher in pancreatitis ($P = 0.01437$), which was anticipated, because MMP-9 is an established inflammation marker of the pancreas.[42] Genetic expression analysis predicted a decreased activity of neutrophil elastase (NE) in tumor tissue. Interestingly and quite contrary to this prediction, NE activity was higher in both pancreatic cancer groups, compared to their respective control groups. Due to the large variations found in both, patient and control groups, statistically significant differences were not discerned. Without further experimental data, it won't be possible to satisfactorily explain the observed discrepancy between gene expression analysis and protease activity measurements.

The results of all protease/arginase activity measurements are summarized in Table 3, which contains the calculated P values for each comparison between the pancreatic cancer (sub-)groups and their age- and gender-matched control groups, as well as for the comparison patients with pancreatitis and pancreatic cancer, and of patients with pancreatitis and their healthy control group. All pairings of pancreatic cancer (sub-)groups and enzymes, for which a significant difference in activity is found ($P < 0.05$), and the enzymatic activity is higher in pancreatic cancer, are shown in green. All pairings of pancreatic cancer (sub-)groups and enzymes, for which a significant difference in activity is found ($P < 0.05$), and the enzymatic activity is lower in pancreatic cancer, are shown in yellow. All pairings for which significant differences were not calculated, are shown in red. They may become significant when more human subjects become available.

In Table 3 the P values[23] obtained for comparisons of the protease/arginase expression pattern in each cancer sub-group with those of the healthy control group are tabulated. The color green denotes measured fluorescence signals that are different from the control group with high significance with an average that is higher than of the control group. ($P < 0.05$). The color yellow denotes measured fluorescence signals that are different from the control group with high significance with an average that is lower than of the control group. ($P < 0.05$). Red denotes all cases where there no statistically significant difference between the cancer (sub)group and the control group were found. Group sizes are: apparently healthy volunteers: n = 48, all pancreatic cancers (PAN): n = 35. pancreatic ductal adenocarcinoma (DAC): n = 9, pancreatitis (PCT): n = 4. All samples were obtained from the Biospecimen Repository Facility of the University of Kansas Cancer Center.[30] ARG: arginase, CTS: cathepsin, MMP: matrix metalloproteinase, uPA: urokinase plasminogen activator, NE: neutrophil elastase.

It is noteworthy that there are more than 300,000 admissions in the US per year because of acute pancreatitis, resulting in yearly treatment costs of about 2 billion US dollars.[43] Common reasons for developing pancreatitis are complications from having gallbladder stones, high triglyceride and calcium blood levels, heavy alcohol consumption, as well as side-effects from taking medication. The mortality rate for acute pancreatitis is about 10 percent. Recurrence is quite frequent (16.5% to 25%) within the first five years.[43] The protease signature that was discovered in this study may also help in detecting early recurrence of pancreatitis. Chronic pancreatitis, a long-term inflammation of the pancreas, is much harder to diagnose than acute pancreatitis, due to the absence of specific symptoms and the fact that the pancreas is a rather inaccessible organ.[44] Chronic pancreatitis, which is prevalent in smokers, patients with increased alcohol consumption and also caused by a gene mutation, is linked with an increased risk of pancreatic cancer.[43] Therefore, patients who have been diagnosed with chronic pancreatitis should be routinely checked for pancreatic cancer. It is our prediction that the liquid biopsy developed here will be suitable to perform this test during routine check-ups.

Conclusions

All pancreatic cancer patients vs. all volunteers

Owing to the robust group sizes of human subjects, (pancreatic cancer patients: n = 35, healthy volunteers: n = 48), arginase,

cathepsins B and E, MMP-1 and -3, and urokinase plasminogen activator were established as suitable biomarkers for pancreatic cancer in serum. In the clinical practice, this means that this panel of enzymes will be able to detect whether a patient potentially has pancreatic cancer by means of a liquid biopsy. Further methods of clinical diagnostics, for instance a CT scan or high-field MRI will then follow to ascertain the patient's clinical condition.

Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinomas comprise more than 90 percent of all pancreatic cancers.[45] They also possess the lowest survival rate.[45] Therefore, detecting them early by means of a simple and affordable liquid biopsy is most desirable. This study has identified two enzymes: MMP-3 and UPA that could detect DAC with high significance even in a relatively small group of human subjects (DAC: n = 9, control group: n = 9). Arginase, cathepsin-B and -E, and MMP-1 are promising candidates for a panel of enzymes that could be used for pancreatic ductal adenocarcinoma detection. A larger confirmatory study is planned for the near future.

Pancreatitis has a different protease signature than both pancreatic cancer patients and healthy patients. Therefore, it can be detected by means of a liquid biopsy and clearly distinguished from pancreatic cancer. Recurrent pancreatitis could be detected as well by performing a serum test.

These findings are potential game-changers in pancreatic cancer detection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2018.04.020.

References

1. http://www.cancer.org/cancer/pancreaticcancer/detailedguide/pancreatic-cancer.
2. Peery AF, Crockett SD, Barritt AS, Dellon ES, Eluri S, Gangarosa LM, et al. Burden of gastrointestinal, liver, and pancreatic diseases in the United States. *Gastroenterology* 2015;**149**(7) [1731-1741.e3].
3. Boeck S, Ankerst DP, Heinemann V. The role of adjuvant chemotherapy for patients with resected pancreatic cancer: systematic review of randomized controlled trials and meta-analysis. *Oncology* 2007;**72**(5–6):314-21.
4. Feig C, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* 2012;**18**(16):4266-76.
5. Elie BT, Gocheva V, Shree T, Dalrymple SA, Holsinger LJ, Joyce JA. Identification and pre-clinical testing of a reversible cathepsin protease inhibitor reveals anti-tumor efficacy in a pancreatic cancer model. *Biochimie* 2010;**92**(11):1618-24.
6. Pancreas, A. J. C. o. C. E. a. E. *AJCC cancer staging manual*. 7th Edition. New York, NY: Springer; 2010241-6.
7. http://www.personalgenome.com/.
8. http://www.genomichealth.com/.
9. https://www.myriad.com/.
10. Morris-Stiff G, Taylor MA. Ca19-9 and pancreatic cancer: is it really that good? *J Gastrointest Oncol* 2012;**3**(2):88-9.
11. Ozkan H, Kaya M, Cengiz A. Comparison of tumor marker CA 242 with CA 19-9 and carcinoembryonic antigen (CEA) in pancreatic cancer. *Hepato-Gastroenterology* 2003;**50**(53):1669-74.
12. Kinugasa H, Nouso K, Miyahara K, Morimoto Y, Dohi C, Tsutsumi K, et al. Detection of K-ras gene mutation by liquid biopsy in patients with pancreatic cancer. *Cancer* 2015;**121**(13):2271-80.
13. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* 2018eaar3247.
14. Wang H, Udukala DN, Samarakoon TN, Basel MT, Kalita M, Abayaweera G, et al. Nanoplatforms for highly sensitive fluorescence detection of cancer-related proteases. *Photochem Photobiol Sci* 2014;**13**(2):231-40.
15. Udukala DN, Wang H, Wendel SO, Malalasekera AP, Samarakoon TN, Yapa AS, et al. Early breast cancer screening using iron/iron oxide-based nanoplatforms with sub-femtomolar limits of detection. *Beilstein J Nanotechnol* 2016;**7**:364-73.
16. Malalasekera AP, Wang H, Samarakoon TN, Udukala DN, Yapa AS, Ortega R, et al. A nanobiosensor for the detection of arginase activity. *Nanomedicine* 2016;**13**(2):383-90.
17. Mason SD, Joyce JA. Proteolytic networks in cancer. *Trends Cell Biol* 2011;**21**(4):228-37.
18. Mondanelli G, Ugel S, Grohmann U, Bronte V. The immune regulation in cancer by the amino acid metabolizing enzymes ARG and IDO. *Curr Opin Pharmacol* 2017;**35**:30-9.
19. Rath M, Muller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Front Immunol* 2014;**5**:1-10.
20. Quesada V, Ordonez GR, Sanchez LM, Puente XS, Lopez-Otin C. The Degradome database: mammalian proteases and diseases of proteolysis. *Nucleic Acids Res* 2009;**37**(Database):D239-43.
21. Choi Y, Kendziorski C. Statistical methods for gene set co-expression analysis. *Bioinformatics* 2009;**25**(21):2780-6.
22. https://www.ncbi.nlm.nih.gov/geo/.
23. Welch BL. The generalisation of student's problems when several different population variances are involved. *Biometrika* 1947;**34**(1–2):28-35.
24. Coffey CS, Cofield SS. Parametric linear models. *Biostatistics* 2006;**15**(DNA microarrays and related genomics techniques):223-43.
25. Page RB, Stromberg AJ. Linear methods for analysis and quality control of relative expression ratios from quantitative real-time polymerase chain reaction experiments. *TheScientificWorldJOURNAL* 2011;**11**:1383-93.
26. https://www.statsdirect.com/help/basics/p_values.htm.
27. Witten D, Tibshirani R. A comparison of fold-change and the t-statistic for microarray data analysis. *Analysis* 1776;**2007**:58-85.
28. Xu C, Xu K, Gu H, Zheng R, Liu H, Zhang X, et al. Dopamine as a robust anchor to immobilize functional molecules on the iron oxide shell of magnetic nanoparticles. *J Am Chem Soc* 2004;**126**(32):9938-9.
29. https://www.ebi.ac.uk/merops/.
30. http://www.kucancercenter.org/patient-care/biospecimen-bank/staff.
31. http://www.cellgrow.com/heat-inactivation/.
32. Doucet A, Butler GS, Rodriguez D, Prudova A, Overall CM. Metadegradomics: toward in vivo quantitative degradomics of proteolytic post-translational modifications of the cancer proteome. *Mol Cell Proteomics* 2008;**7**(10):1925-51.
33. Rakashanda S, Rana F, Rafiq S, Masood A, Amin S. Role of proteases in cancer: a review. *Biotechnol Mol Biol Rev* 2012;**7**(4):90-101.
34. Bossmann SH, Wang H. *Magnetic Nanomaterials: Applications in Catalysis and Life Sciences*. London: The Royal Society of Chemistry; 2017268. ISBN: 978-1-78262-788-3.
35. Lacroix L-M, Frey Huls N, Ho D, Sun X, Cheng K, Sun S. Stable single-crystalline body centered cubic Fe nanoparticles. *Nano Lett* 2011;**11**(4):1641-5.
36. Wang H, Shrestha TB, Basel MT, Dani RK, Seo G-M, Balivada S, et al. Magnetic-Fe/Fe3O4-nanoparticle-bound SN38 as carboxylesterase-cleavable prodrug for the delivery to tumors within monocytes/macrophages. *Beilstein J Nanotechnol* 2012;**3**:444-55 12 pp.
37. Sobczynski J, Toennesen HH, Kristensen S. Influence of aqueous media properties on aggregation and solubility of four structurally related

meso-porphyrin photosensitizers evaluated by spectrophotometric measurements. *Pharmazie* 2013;**68**(2):100-9.
38. Patsenker L, Tatarets A, Kolosova O, Obukhova O, Povrozin Y, Fedyunyayeva I, et al. Fluorescent probes and labels for biomedical applications. *Ann N Y Acad Sci* 2008;**1130**(Fluorescence methods and applications):179-87.
39. Ben-Avraham D, Schulman LS, Bossmann SH, Turro C, Turro NJ. Luminescence quenching of ruthenium(II)-Tris(phenanthroline) by cobalt(III)-Tris(phenanthroline) bound to the surface of starburst dendrimers. *J Phys Chem B* 1998;**102**(26):5088-93.
40. Rukamp B, Powers JC. *Cathepsins*. Taylor & Francis Ltd.; 200284-126.
41. Piperi C, Papavassiliou AG. Molecular mechanisms regulating matrix metalloproteinases. *Curr Top Med Chem* 2012;**12**(10):1095-112.
42. Al Mofleh IA. Severe acute pancreatitis: pathogenetic aspects and prognostic factors. *World J Gastroenterol* 2008;**14**(5):675-84.
43. https://pancreasfoundation.org/patient-information/acute-pancreatitis/acute-pancreatitis-diagnosis-and-treatment/.
44. Banks PA, Conwell DL, Toskes PP. The management of acute and chronic pancreatitis. *Gastroenterol Hepatol* 2010;**6**(2 Suppl 5):1-16.
45. http://www.cancer.gov/cancertopics.